Am. J. Hum. Genet. 62:1062–1069, 1998

Localization of the Gene for Congenital Dyserythropoietic Anemia Type I to a <1-cM Interval on Chromosome 15q15.1-15.3

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Summary

Congenital dyserythropoietic anemias (CDA) are a rare group of red-blood-cell disorders of unknown etiology that are characterized by ineffective erythropoiesis, pathognomonic cytopathology of the nucleated red blood cells in the bone marrow, and secondary hemochromatosis. In CDA type I, bone-marrow electron microscopy reveals characteristic findings in erythroid precursors, including spongy heterochromatin and enlarged nuclear pores. Since the genetic basis of CDA type I is not evident, we used homozygosity and linkage mapping to localize the genetic defect responsible for CDA type I in 25 Bedouins from four large consanguineous families. We report the linkage of this disease to markers on chromosome 15 located at q15.1-q15.3. Fourteen markers within a 12-cM interval were typed in the relevant family members. Nine of the markers yielded maximum LOD scores of 1.625–12.928 at a recombination fraction of .00. Linkage disequilibrium was found only with marker D15S779. Haplotype analysis revealed eight different carrier haplotypes and highlighted the existence of a founder haplotype. Identification of historical crossover events further narrowed the gene location to between D15S779 and D15S778. The data suggest localization of the CDA type I gene within a 0.5-cM interval. The founder mutation probably occurred ⩾400 years ago. Sequence analysis of the coding region of protein 4.2, the only known erythroid-specific gene in the locus, did not reveal any change in the CDA type I patients. Future analysis of this locus may lead to the identification of a gene essential to normal erythropoiesis.

Received December 8, 1997; accepted for publication March 2, 1998; electronically published April 17, 1998.

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0002-9297/98/6205-0010$02.00

Introduction

Congenital dyserythropoietic anemias (CDA) are a rare group of red-blood-cell disorders of unknown etiology that are characterized by ineffective erythropoiesis, pathognomonic cytopathology of the nucleated red blood cells in the bone marrow, and secondary hemochromatosis. In 1968, Heimpel and Wendt classified these disorders into three types, which were later confirmed by electron microscopy and serological findings (Alter and Young 1993). Type I (MIM 224120) is associated with moderate congenital macrocytic anemia, megaloblastoid erythroid hyperplasia, and the presence of nuclear-chromatin bridges between erythroblasts. Bone-marrow electron microscopy reveals characteristic findings in erythroid precursors, including spongy heterochromatin, enlargement of the nuclear pores, and occasional invagination of cytoplasmic organelles into the nuclear area. The underlying mechanisms for those abnormalities are unknown. We recently demonstrated that, in CDA type I, the ultrastructural morphological features of the erythroid precursors resemble apoptosis, and S-phase arrest is also found (Tamary et al. 1996).

More than 70 sporadic cases of CDA type I have been reported (Wickramasinghe 1997). We recently described a cluster of 20 Israeli Bedouin patients with the disease (Tamary et al. 1996). The pattern of inheritance is autosomal recessive (Heimpel and Wendt 1968). We showed that 55% of the patients manifested at the neonatal period with anemia and jaundice and that 88% of those neonates required blood transfusions during the 1st mo of life (Shalev et al. 1997). Few patients display patches of brown skin pigmentation and congenital skeletal abnormalities (Wickramasinghe 1997). Later in life, jaundice, splenomegaly, and moderate macrocytic anemia are usually present. The condition is compatible with normal survival but carries an increased risk of gallstone development, iron overload, and secondary hemochromatosis. Only a few patients are more severely affected and are transfusion dependent (Heimpel 1998).

The more common CDA type II, also known as "hereditary erythroblastic multinuclearity with a positive acidified serum" (HEMPAS) test (Lewis and Frisch 1973), is an autosomal recessive disorder manifested by normocytic anemia, variable jaundice, and hepatosplenomegaly. Bone-marrow aspiration disclosed bi- and multinucleated erythroblasts and karyorrhexis. Biochemical data suggested that this disease is due to a deficiency of either N-acetylglucoaminyltransferase II or α-mannosidase II (Fukuda et al. 1992). However, recent linkage analysis has excluded, as candidate genes, those genes encoding these proteins, and the CDA type II gene has been mapped to chromosome 20 (20q11.2) (Gasparini et al. 1997; Iolascon et al. 1997).

CDA type III is associated with anemia, jaundice, and occasional splenomegaly and is identified by the presence of macrocytosis and bone-marrow erythroblastic multinuclearity with prominent gigantoblasts (Alter and Young 1993). The pattern of inheritance is autosomal dominant. The defect in CDA type III was recently localized to chromosome 15q21-q22 (Lind et al. 1995).

The autosomal recessive inheritance of CDA type I and the consanguineous nature of the Bedouin families make these families suitable candidates for homozygosity mapping (Lander and Botstein 1987). In the present study, we used homozygosity linkage mapping to localize the genetic defect responsible for CDA type I in four consanguineous Bedouin families. We demonstrate the close linkage of CDA type I to microsatellite markers on chromosome 15 (15q15.1-15.3). Moreover, the carrier chromosome haplotype revealed the existence of an ancestor haplotype and a possible major mutation in the Bedouin population. The age of the mutation has been estimated.

Families and Methods

Families

The 25 CDA type I patients investigated (most of whom have been described elsewhere [Tamary et al. 1996]) are part of four large consanguineous Bedouin families (fig. 1). Diagnosis was confirmed by typical bone-marrow–erythroid electron-microscopy findings, in all cases.

DNA Extraction

Blood samples were obtained from the 25 affected patients and from 45 other family members (fig. 1). DNA was prepared from peripheral blood leukocytes as described elsewhere (Poncz et al. 1982). All subjects gave their informed consent.

Genotype Analysis

Primer pairs for the detection of 157 polymorphic markers were obtained from Research Genetics (Human Screening Panel, version 5a). These markers consist of highly polymorphic short tandem repeats (STRs), mostly tri- and tetranucleotide repeats, developed by the Cooperative Human Linkage Center. The average heterozygosity of those markers is .8.

PCR amplification was performed in a final volume of 15 μl containing 45 ng of purified genomic DNA, 10 mM Tris-HCl pH 8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 μM of each dNTP, 0.25 U of *Taq* polymerase (Perkin Elmer Cetus), and 3 pmol of each primer. Amplification was performed for 34 cycles, each with denaturation at 94°C for 30 s, annealing at 54°C–59°C for 1 min, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The amplified products were separated by electrophoresis on a 5%–15% denaturating polyacrylamide gel. Allele sizes were determined by comparison with *Msp*I-digested pBR322, which was included as a size standard in the gels. All markers were scored independently by two observers; the scores were compared, and any discrepancies were resolved by reexamination of the gels.

In an attempt to pinpoint the CDA type I gene location, additional STR markers (Allamand et al. 1995; Chainnilkulchai et al. 1995; Richard et al. 1995) mapping to the region of interest (15q15.1-15.3) were used. Their primer sequences were obtained from the Genome Database via URL http://gdbwww.gdb.org/. DNA samples from all affected individuals and their family members were typed for the additional markers.

Statistical Analysis

LOD scores between the gene for CDA type I and the various markers were calculated with the LIPED program, version 5.0 (Ott 1974). An autosomal recessive model with complete penetrance in both sexes, a frequency of .005 for the disease allele, and equal allele frequencies for each marker were assumed. The relevant family members were fully typed for all loci, making the results insensitive to allele frequencies. Specific allele frequencies in our population were also estimated by calculation of their frequency of occurrence in the noncarrier chromosome.

The linkage disequilibrium of each microsatellite with the CDA type I locus was examined by comparison of marker-allele frequencies in CDA type I chromosomes versus marker-allele frequencies in normal chromosomes, by χ^2 test with the $2 \times K$ contingency table, where K is the number of unique marker alleles present in the sample. The significance of the association was determined by a right-sided χ^2 test with $K - 1$ df.

The maximum-likelihood technique was applied to

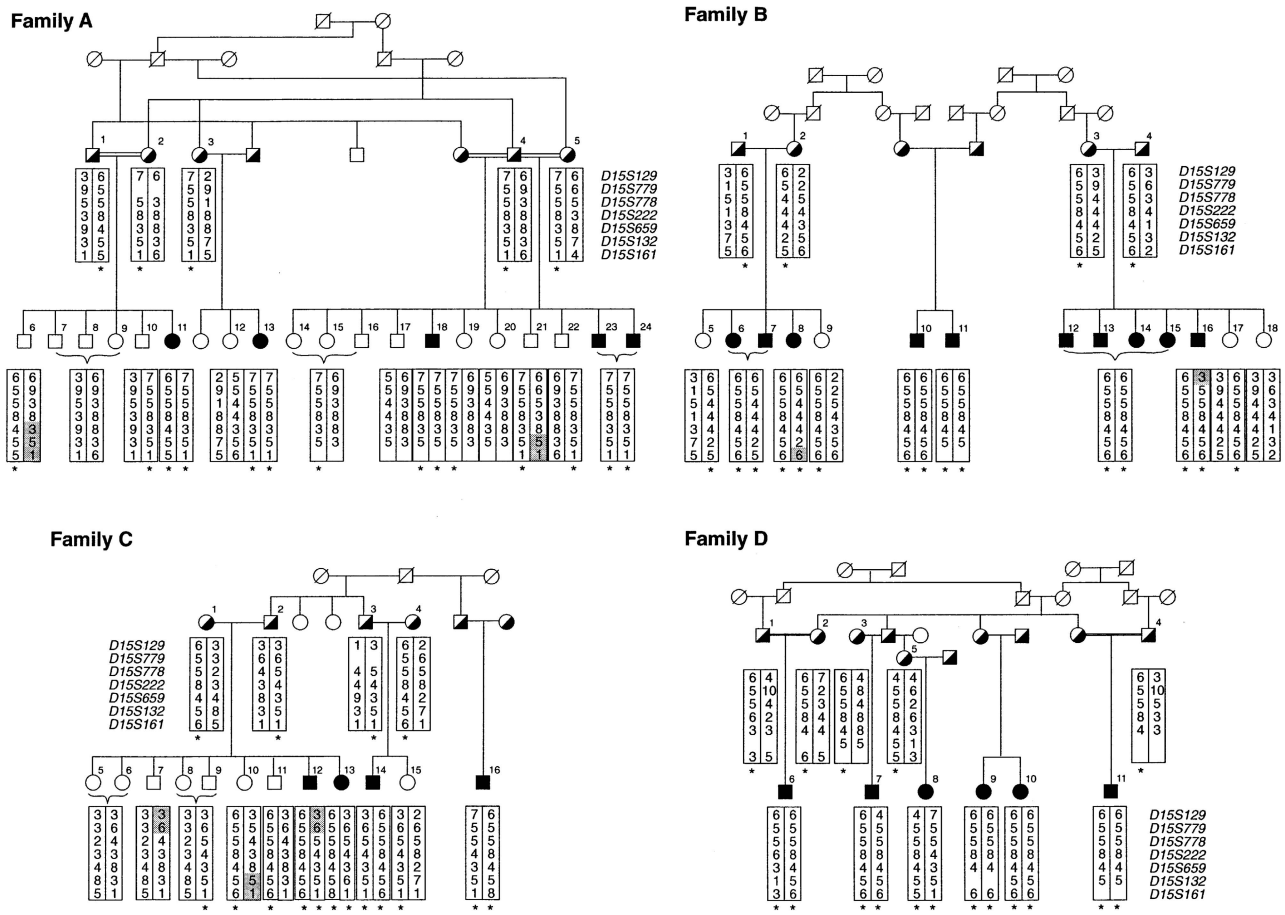

Figure 1 Pedigree of four CDA type I Bedouin families. Affected family members are denoted by blackened symbols; males are denoted by squares; and females are denoted by circles. The most likely haplotype for the chromosome 15 markers is shown below each family member for whom DNA was available. The order of the markers, from the top to the bottom of each bar, is D15S129, D15S779, D155S778, D15S222, D15S659, D15S132, and D15S161. Carrier haplotypes are denoted by asterisks (*); and noninformative regions are denoted by gray shading.

estimate the number of generations, *g,* that have elapsed since the origin of a given haplotype from one common chromosome (Bonne-Tamir and Korostishevsky 1997). The length of the common segments flanking the putative mutation site was measured for each haplotype pair among the 15 unrelated obligate-carrier haplotypes identified in the population. The basic assumptions were that the haplotypes are derived from the same mutant chromosome and that the length of the intact interval is limited by recombination events only. The support interval for *g* was based on a 1-LOD-unit support interval for each of the recombinations (Risch et al. 1995).

Automated DNA Sequence Analysis

The genomic organization of the protein 4.2 gene has been reported elsewhere (Korsgren and Cohen 1991). Amplified functional regions of the gene (promoter and exons, including exon-intron borders) were sequenced by fluorescence-based DNA sequence analysis, by use of a *Taq* Dye Terminator Cycle Sequencing Kit on a Prism 377 DNA sequencer (Applied Biosystems).

Results

Linkage Analysis

A genomewide search for regions of homozygosity by descent, by means of STR markers, was conducted with use of DNA from 25 Bedouins affected with CDA type I. The first marker exhibiting homozygosity was located on chromosome 15q (D15S659) and was found in 14 individuals. Genotyping of 45 unaffected individuals representing the parents and siblings of the affected individuals in the four families revealed only 4 individuals who were homozygous for this marker.

An additional 13 markers were then examined; the maximum LOD score (12.928) was later obtained with D15S778. Linkage of adjacent markers confirmed localization of the CDA type I gene to region

Table 1

Pairwise LOD Scores between Chromosome 15 Markers and the CDA Type I Locus

Marker	LOD Score at Recombination Fraction of							Maximum LOD Score	Estimated Recombination Fraction
	.000	.001	.050	.100	.200	.03	.04		
D15S129	−∞	3.271	5.181	4.787	3.247	1.907	.586	5.190	.04
D15S1044	−∞	1.725	3.943	3.688	8.626	1.450	.456	3.943	.05
D15S514	9.718	9.695	8.533	7.351	5.044	2.909	1.094	9.718	.00
D15S779	11.815	11.837	11.135	9.842	7.004	4.166	1.632	11.837	.00
D15S780	8.237	8.218	7.287	6.325	4.402	2.572	1.001	8.237	.00
D15S515	1.625	1.620	1.349	1.096	.665	.344	.127	1.625	.00
D15S778	12.928	12.900	11.533	10.108	7.211	4.358	1.776	12.928	.00
D15S784	10.901	10.876	9.617	8.318	5.724	3.259	1.184	10.901	.00
D15S783	10.284	10.261	9.131	7.982	5.664	3.411	1.345	10.284	.00
D15S781	5.881	5.815	5.046	4.271	2.768	1.406	.387	5.831	.00
D15S222	6.393	6.381	5.728	5.017	3.509	1.985	.650	6.393	.00
D15S659	−∞	4.860	5.585	4.903	3.313	1.772	.525	5.783	.02
D15S132	−∞	1.262	5.325	5.155	3.878	2.255	.740	5.350	.06
D15S161	−∞	−1.220	2.350	2.442	1.840	1.035	.319	2.47	.08

D15S514–D15S222 on the proximal part of 15q15.1-15.3 (table 1). The order and distance of additional flanking markers were calculated by marker-to-marker linkage analysis (data not shown).

All informative recombinations are presented in figure 2. Two key recombinant events were observed, one in family A (member A-6) and the other in family C (member C-12). In A-6, recombination had occurred between the gene for CDA and D15S659, localizing the gene as telomeric to D15S222. Recombination in C-12 localized the gene as centromeric to D15S1044. The integrated data from all the recombination events showed that the candidate region lies between markers D15S659 and D15S1044.

Linkage Disequilibrium

At total of 26 different haplotypes were evaluated, including 18 "normal" chromosomes and 8 carrier chromosomes (table 2). Chromosomes either shared by two siblings or from a common consanguineous origin were counted only once. Allelic association between the CDA type I gene and each of the markers, as determined by χ^2 test, revealed a marked association with the D15S779 marker ($\chi^2 = 20.25$, $P = .005$; see table 3). Allele 5 in marker D15S779 was observed in 7 of 8 different carrier haplotypes and in 0 of 18 non-carrier haplotypes. No other marker showed any significant association.

Haplotype Analysis

The haplotypes of the affected individuals and their available parents and siblings were then analyzed to identify informative historical crossover events that would permit finer localization of the gene for CDA type I (fig. 1). Eight different carrier haplotypes were constructed, by use of 14 markers, for affected family members (table 2). A comparison between the carrier haplotypes revealed the existence of historical recombinant events and narrowed the CDA type I location to between D15S779 and D15S778, as the common interval for all the carrier haplotypes (table 2). Allele 4 in marker D15S781 and allele 2 in marker D15S515 were common to all the haplotypes. However, since allele changes can result from mutations, particularly when only a single variant allele is buried in the middle of a conserved hap-

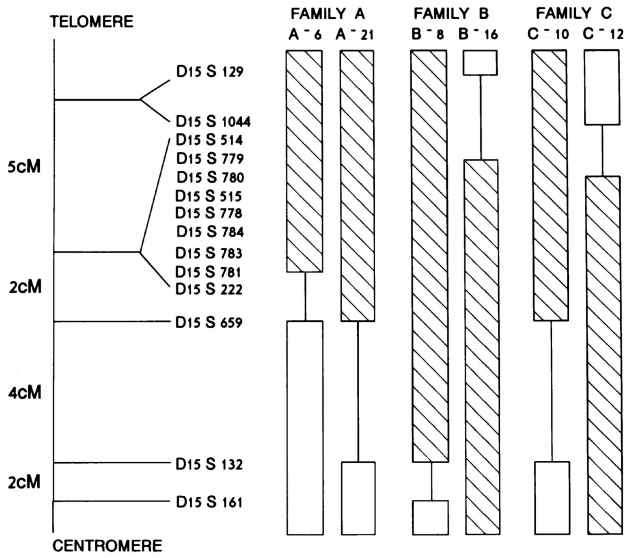

Figure 2 All informative recombinations between the gene for CDA type I and genetic markers on chromosome 15. The microsatellite map was inferred from the study by Allamand et al. (1995) and the present study (table 2). Portions of chromosomal regions containing the gene for CDA type I are denoted by hatching; vertical lines denote noninformative regions; and bars without hatching denote regions that do not include the gene.

Table 2

Eight Carrier Haplotypes Segregating within the Families with CDA Type I

	Allele at Locus[a]														
Haplotype	D15S129	D15S1044	D15S514	D15S779	D15S780	D15S515	D15S778	D15S784	D15S783	D15S781	D15S222	D15S659	D15S132	D15S161	Families[b]
I	6	3	1	5	4	2	5	4	1	4	8	4	5	6	B (3), C (3), D (2)
II	6	3	1	5	4	2	5	4	4	4	6	3	1	3	D (1)
III	6	3	1	5	4	2	4	5	2	4	4	4	2	5	B (1)
IV	4	1	1	5	4	2	5	4	1	4	8	4	5	5	D (1)
V	7	2	1	5	4	2	5	4	1	4	8	3	5	1	A (1)
VI	7	2	1	5	4	2	5	4	1	4	4	3	5	1	C (1), D (1)
VII	3	3	1	6	4	2	5	4	1	4	4	3	5	1	C (1)
VIII	6	3	1	5	4	2	5	4	1	4	8	4	5	5	A (1)

[a] Data within the "frame" are identical to the common haplotype.

[b] Numbers in parentheses are number of unrelated haplotypes in same family.

Table 3

Allelic Association between the CDA Type I Gene and Markers on Chromosome 15q15

Locus	χ^2 [a]	df	*P*	Heterozygosity[b]
D15S129	8.34	5	.141	.77
D15S1044	7.00	4	.135	.64
D15S514	6.62	4	.159	.63
D15S779	2.25	7	.005	.82
D15S780	5.54	4	.237	.67
D15S515	1.26	1	.262	.23
D15S778	6.32	4	.196	.73
D15S784	5.37	5	.393	.69
D15S783	3.76	2	.153	.55
D15S781	7.84	4	.097	.64
D15S222	4.89	5	.429	.75
D15S659	6.91	5	.228	.77
D15S132	11.2	5	.048	.76
D15S161	1.48	5	.915	.75

[a] Between 8 different carrier haplotypes and 18 different noncarrier parental haplotypes.

[b] Based on allele frequencies in 18 different noncarrier parental haplotypes.

lotype (e.g., allele 6 in marker D15S779 and allele 4 in marker D15S778; see table 2), the actual interval can be between D15S514 and D15S784.

A total of 16 unrelated obligate carrier haplotypes were identified in the families studied. Two groups of haplotypes sharing either all the alleles or all except one allele were identified. The first group of haplotypes, evenly distributed in all the families, contains eight of haplotype I and one of haplotype VIII. The second group of haplotypes contains one of haplotype V and two of haplotype VI. The common region between these two groups of haplotypes includes an interval of ~7 cM between D15S1044 and D15S222, which is preserved in 13 (81%) of the 16 obligate-carrier haplotypes.

Another four haplotypes were found in a single copy each: haplotypes II and IV in family D, haplotype III in family B, and haplotype V in family A. Haplotypes II–IV most likely resulted from a single recombinant event involving a haplotype of the first group, whereas haplotype VII is the result of a single recombination event in the second group.

According to our calculation, we estimate *g* as being 32.18 (range 17.68–49.74), with the support interval based on 1 LOD unit (Risch et al. 1995).

Sequence Analysis of the Protein 4.2 Gene

No change in the sequence of the protein 4.2 gene (Korsgren and Cohen 1991) was detected by automatic sequencing of the amplified promotor region (385 bp upstream of the initiation codon), exons, and exon-intron borders (exons 1–13) in the four patients we examined.

Discussion

CDA type I is a rare autosomal recessive disease characterized by moderate macrocytic anemia with typical electron-microscopy findings in erythroid precursors, including spongy heterochromatin and enlargement of nuclear pores (Lewis and Frisch 1973). Although most patients suffer from moderate anemia, a few are more severely affected and are transfusion dependent. In a previous report, we have suggested that the erythroid bone-marrow electron-microscopy findings resemble apoptosis, whereas the S-phase arrest, also described in those precursors, may be secondary to the observed apoptosis (Tamary et al. 1996). The basic defect in this disease remains unknown.

The gene for CDA type II recently has been localized to chromosome 20q11.2 (Iolascon et al. 1997), and the gene for CDA type III has been localized to chromosome 15q21-15q22 (Lind et al. 1995). Our patients, however, revealed no linkage to those markers.

Homozygosity-mapping analysis in Israeli Bedouins with CDA type I suggests that the gene for CDA type I is localized to an ~7-cM area on chromosome 15q15.1-15.3. Additional genetic information was obtained by linkage-disequilibrium and haplotype analysis. Although linkage disequilibrium alone may be misleading if data are derived from a single population (Allamand and Beckmann 1997), the combined strategy allowed us to localize our gene to an interval of ~ 0.5 cM between markers D15S779 and D15S778, corresponding in this region to 1.3–1.6 Mb (Chiannilkulchai et al. 1995). However, if microsatellite divergence is due to mutation, the interval many be longer—that is, between D15S514 and D15S784. The CDA type I locus is ~20 cM centromeric to the locus for CDA type III.

While searching for the gene for the recessive form of limb-girdle muscular dystrophy 2A, Chiannilkulchai et al. (1995) generated a primary-muscle–expression map of chromosome 15q15 and revealed 17 sequences corresponding to 5 known genes and 12 new ones. In a previous report, erythrocyte membrane protein 4.2 (EPB4.2) has been localized to 15q15 (Najfeld et al. 1992). EPB4.2 is a membrane protein that helps to bind the red-blood-cell cytoskeleton to the lipid bilayer, probably by anchoring it to ankaryin. EPB4.2 deficiency is associated with congenital spherocytosis (Gallagher et al. 1998). Therefore, it was not considered, a priori, to be a candidate gene for CDA type I. However, since EPB4.2 is the only erythroid-specific gene in the CDA type I interval, we consider it a candidate, assuming that this protein has some unknown properties. However, sequence analysis of functionally important regions in the EPB4.2 gene did not disclose any changes in sequence, thus decreasing the likelihood of involvement of EPB4.2 in CDA type I.

Close examination of the eight carrier haplotypes found among our CDA type I patients suggests that all of the haplotypes share at least two markers, D15S515 and D15S780, whereas 81% of them share a larger, 7-cM, ancestor haplotype, D15S2044–D15S659, with possible recombinations in the flanking regions (table 2). These data point to a single founder mutation for most of the carrier haplotypes that we studied.

The Bedouins living in Israel originated mainly in the Arabian peninsula; the few members who were not originally nomads joined them at a later stage (Marx 1967, pp. 3–31). Our patients are members of what seem to be four unrelated families. However, no written history is available, and there is no objective way of determining their exact ethnic origin. On the basis of our data, all four consanguineous families apparently stem from a common ancestor estimated to have lived ~400–500 years ago.

The data provided here may serve as the starting point for identification of the gene responsible for CDA type I. They should also provide further insight into normal and abnormal erythroid differentiation.

Acknowledgments

This work was supported by the J. Maus and G. Ceaseman-Maus Chair in Pediatric Hematology (R. Zaizov, incumbent). The authors are grateful to J. Zlotogora, for his important suggestions, and to J. Beckman, for his valuable scientific discussion and critical review of the manuscript. We thank Alexandra Mahler for her skillful assistance in preparation of the manuscript.

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